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+IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Michael A. Apicella et. al
Serial No. : 10/066,551
Filed : January 31, 2002
Title : VACCINE AND COMPOUNDS FOR THE PREVENTION AND TREATMENT OF NEISSERIAL INFECTONS

Art Unit : 1645
Examiner : P. Baskar

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MICHAEL A. APICELLA UNDER 37 C.F.R. § 1.132

I, Michael A. Apicella, hereby declare:

1. I am one of the co-inventors of the claims in the above-captioned patent application. I am currently a tenured Professor and Chairman of the Department of Microbiology at the University of Iowa in Iowa City, Iowa. I have held that position for over ten years. I began my scientific career at Johns Hopkins University in 1966 and since that time have been a faculty member of the State University of New York at Buffalo and the University of Nevada, Reno. I obtained my M.D. degree from the State University of New York in Brooklyn in 1963. I have worked in the area of bacterial pathogenesis and genetics for the past 35 years after completing my post-doctoral fellowship at Johns Hopkins School of Medicine. I have published over 160 articles in peer reviewed scientific journals in these areas since that time.

2. In an Office Action dated July 30, 2003, claims 15, 19 and 21 were rejected as not being enabled for providing a protective immune response against gonorrhea disease caused by *Neisseria gonorrhoeae*.

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

January 30, 2004
Date of Deposit

BL Taylor
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Brenda L. Taylor
Typed or Printed Name of Person Signing Certificate

3. We carried out experiments to show the method of cervical infection of cervical epithelia. Tissue culture media was removed from primary cervical cell cultures at least 24 h prior to cell gonococcal challenge, and it was replaced with medium (RPMI 1640) lacking antibiotics. Antibiotic-free tissue culture medium from primary cervical cell cultures served as the source for alternative complement pathway components for all the assays. For infection studies bacteria were allowed to grow overnight (37°C, 5% CO_2) on GC-IsoVitaleX agar plates prior to harvesting with a sterile swab and resuspending in sterile physiological saline. Bacterial culture density was determined spectrophotometrically where an optical density of 1 at 600 nm was equivalent to 10^9 bacteria ml^{-1}. Cultures of gonococci were further diluted in antibiotic-free RPMI 1640 to a density of 10^8 bacteria ml^{-1}. 10^7 gonococci were then used to infect cervical cell monolayers at a multiplicity of infection of 100. Infection was allowed to progress for the designated time after which the infection medium was removed, and the cell monolayers were extensively washed with phosphate-buffered saline (PBS). Uninfected, control cell monolayers were simultaneously processed with challenged cell monolayers.

4. An antibody specific to PLD was made and verified in the following manner. The antibody 1307 was made by immunizing rabbits with the peptide RRMHNLSFTADNR. This peptide represents the first active site region (amino acids 181-193) of the enzyme. The specificity of the antibody was confirmed by Western blot analysis, which demonstrated binding of 1307 to a 55kDA protein and the ability of this antibody to inhibit enzymatic active of PLD in the Amplex-red PLD Assay (Molecular Probes, Eugene, OR Cat#A-12219).

5. The results indicated an inhibition of *N. gonorrhoeae* association and invasion. (*See* Table 1 below.) Primary cervical cell monolayers were infected with gonococci as outlined above. An anti-phospholipase D (PLD) antibody 1307 diluted 1:20 in RPMI1640 was added simultaneously with gonococci as noted above. After one hour of exposure to the primary human cervical epthelial cells, the ability of gonococci to adhere to and/or invade primary ecto- and endocervical cells was quantitatively determined using standard gentimicin-resistance assays, performed as described previously (Edwards, J.L., Shao, J.Q., Ault, K.A., and Apicella, M.A. (2000). Neisseria gonorrhoeae elicits membrane ruffling and cytoskeletal rearrangements

upon infection of primary human endocervical and ectocervical cells. *Infect. Immun.* 68:5354-5363). The total association (*i.e.*, adherence and invasion) of gonococci with primary ecto- and endocervical cells was quantitated by the omission of gentimicin from the above described invasion assay. Inhibition of gonococcal attachment and/or invasion was determined as a normalized function of the ability of gonococci to attach to and/or invade primary endo- and ectocervical cells in the absence of the inhibitors used (*i.e.*, antibody 1307). Percent invasion of *N. gonorrhoeae* strain 1291 was determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate. A Kruskal-Wallis non-parametric analysis of variance was used to determine the statistical significance of the association and invasion assays described above.

6. Treatment with the antiserum interferes with gonococcal association with the cervical epithelial cell with a reduction of approximately 70% (mean 28.13 to 7.92). There is no change in association of the PLD mutant in the presence or absence of antiserum (mean 14.84 to 14.37).

7. Treatment with the antiserum interferes with gonococcal invasion of the cervical epithelial cell with a reduction of approximately 70% (mean 2.72 to 0.81). There is no change in association of the PLD mutant in the presence or absence of antiserum (mean 0.35 to 0.36).

8. Gonococcal PLD is a secreted protein whose secretion is only stimulated in the presence of human cervical epithelial cells. Since gonococci do not contain a known secretion system, the most likely mechanism for release of this protein is via cleavage of a leader sequence on the protein. Sequence analysis using the LipoP analysis aligorithm indicates that there is a protease cleavage site between amino acids 16 and 17 in the PLD molecule. This analysis confirms our view that PLD is a secreted protein.

9. That I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Dated: 1-30-04

Michael Apicella

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Table 1. Effect of an anti-PLD antiserum 1307 on N. gonorrhoeae strain 1291 association with and invasion of Primary Human Cervical ectocervical cells

	Assay 1	Assay 2	Assay 3	Mean	Variance
	Association[1]				
WT1291	28.47	28.4	27.53	28.13	0.42
WT + 1307[2]	9.01	7.21	7.55	7.92	0.78
1291DPLD	14.1	14.26	16.17	14.84	0.94
1291DPLD +1307	13.2	14.1	15.8	14.37	1.08
	Invasion[3]				
WT1291	2.7	2.6	2.9	2.72	0.12
WT + 1307	0.81	0.75	0.83	0.80	0.03
1291DPLD	0.33	0.37	0.35	0.35	0.02
1291DPLD +1307	0.31	0.37	0.39	0.36	0.03

[1] - Percent of inoculum associated with primary primary human cervical ectocervical cells after 60 minutes of infection

[2] - antiserum 1307 (rabbit anti-PLD antiserum) - final dilution 1:20

[3] - Percent of inoculum invading primary primary human cervical ectocervical cells after 60 minutes of infection